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In vitro anthelmintic activity of methanol extracts and fractions of two amphilophium species against *Eisenia Fetida*

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ABSTRACT

This work aimed to determine the chemical composition and evaluate the anthelmintic activity of the methanol extracts and fractions from *Amphilophium paniculatum* and *Amphilophium crucigerum* against *Eisenia fetida*. A preliminary phytochemical analysis was performed to assess the presence of groups of secondary metabolites. The plants were extracted with methanol to obtain the crude extracts. The extracts were submitted to partition with solvents of increasing polarity to obtain the corresponding fractions. The methanolic extracts and the fractions obtained were tested for anthelmintic activity against *E. fetida*, using albendazole as a positive control. The phytochemical test demonstrated the presence of flavonoids, saponins, and steroids/triterpenes for *A. paniculatum* and alkaloids, tannins, saponins, and steroids/triterpenes for *A. crucigerum*. The extracts and fractions of both plants showed a statistically significant decrease of the times of paralysis and death compared to albendazole. The results obtained showed that the methanolic extracts and fractions of *A. paniculatum* and *A. crucigerum* contain compounds that possess anthelmintic activity. The isolation of the substances responsible of the biological effect described could result in the development of new drugs to treat helminth diseases.

1. INTRODUCTION

Soil-transmitted helminthiases are among the most prevalent human diseases throughout the world, affecting primarily large populations of the developing countries, mainly those living in poverty and with difficult access to appropriate sources of drinking water [1]. These infections affect primarily to children and produce negative effects on their cognitive development and education [2].

The parasites also affect agricultural productivity of adults and promote school absenteeism of children. The main regions affected are the Americas, China, East Asia, and sub-Saharan Africa [3]. To fight these diseases, the WHO strategy is based in the administration of an anti-parasitic drug for the children in scholar age that live in the areas with risk of infection.

The main anthelmintic drug used for the treatment is albendazole. This compound belongs to the benzimidazole group and has also activity against some protozoa. Nevertheless, adverse reactions to the drug have been described, among them abdominal pain, nausea, vomiting, diarrhea, headache, dizziness, and vertigo [4]. Dermatological hypersensitivity reactions and alopecia had been reported, although in rare cases. The compound can also produce mild-to-moderate elevation of hepatic transaminases and in rare cases hepatotoxicity [5]. Leucopenia and less frequently granulocytopenia and thrombocytopenia have also been reported [6].

Besides those adverse reactions, another risk is the possible development of resistance by the parasites due to the massive use of the drug. This fact has been already observed in case of the livestock [7].

The use of plants for the treatment of the various illnesses that afflict mankind is known since ancient times. Different classes of compounds have been isolated and those compounds confers a wide range of biological activities to the plants where they are present, one of which is anthelmintic [3].

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The genus *Amphilophium* belongs to the Bignoniaceae family. Triterpene glycosides, aliphatic alcohol glycosides, flavonoids, and sesquiterpenes have been isolated and the two last ones have showed to possess anthelmintic activity, make this genera candidate for the search of this kind of compounds [8–11]. *Amphilophium paniculatum* and *Amphilophium crucigerum* are species that are widely distributed from Mexico to Argentine and can be found in Paraguay and to the best of our knowledge were never evaluated for that activity.

According to these antecedents, we tested the anthelmintic activity of *A. paniculatum* and *A. crucigerum* against *Eisenia fetida*. The selection of this worm as a model is due to its anatomical and physiological similarities with those responsible of human infections [12–14]. We also determined the chemical composition of both plants by preliminary phytochemical analysis, in order to establish groups of secondary metabolites present in them.

2. MATERIALS AND METHODS

2.1. Plant material

Amphilophium paniculatum (L.) Kunth aerial parts were collected in Caacupé, Cordillera Department, Paraguay, whereas *Amphilophium crucigerum* (L.) L.G. Lohmann aerial parts were collected in Capiatá, Central Department, Paraguay. Both species were identified by the researchers from the Department of Botany of the Faculty of Chemical Sciences. Voucher specimens of both plants were deposited in the Herbarium FCQ of the Faculty of Chemical Sciences for indexing purposes (R. Degen 4224 *et* L. Britos, G. González for *A. paniculatum* and L. Britos 222 *et* E. López for *A. crucigerum*).

2.2. Reagents and chemicals

All chemicals were of analytical grade (E. Merck, Darmstadt, Germany), except the methanol used for the preparation of extracts, that was of HPLC grade (J.T. Baker, Center Valley, PA). Albendazole (100% purity) was obtained from a local pharmaceutical company.

2.3. Preparation of the extracts

The aerial parts of *A. paniculatum* and *A. crucigerum* were ground in a cutter mill to fine powder. The ground material (500 g) was poured into a beaker and methanol was added until all the powder was soaked. It was then submitted to an ultrasonic bath for 1 hour and the process was repeated three times with an interval of 15 minutes between them. The entire procedure was repeated during 3 days. The extracts were then filtered through defatted cotton and the solvent was eliminated using a rotary evaporator (RVO 400 SD, Boeco, Germany) to obtain the crude extracts.

2.4. Phytochemical preliminary test

The crude extracts were analyzed by the methods described by Sanabria-Galindo [15] that uses coloration and/or precipitation reactions along with thin-layer chromatography in order to identify the groups of secondary metabolites.

2.5. Fractions preparation

Approximately, 1 g of crude extract was dispersed in water and extracted successively with hexane, chloroform, ethyl acetate, and 1-butanol. The extractive process was repeated three times with each organic solvent. The immiscible organic layers for each solvent were reunited and dried with anhydrous Na_2SO_4. After filtration, the organic solvents were evaporated using a rotary evaporator and the remained aqueous fraction was lyophilized (Labconco Freezone 4.5, USA). The entire process yielded five fractions: hexanic, chloroformic, ethyl acetate, butanolic, and aqueous for both plants.

2.6. Anthelmintic assay

The anthelmintic assay was performed using the Californian red worm *E. fetida*, according to the method described by Pawar *et al*. with some modifications [14]. The worms were from the Phytochemistry Department hatchery and had a length of 4 to 6 cm with a diameter of 0.2 to 0.4 cm. The assays were made in triplicate.

2.7. Statistical analysis

The data were processed according to the D'Agostino & Pearson test to determine if they were normally distributed, followed by analysis of variance (ANOVA) and then by the Tukey method for relevant multiple comparisons using GraphPad Prism software v. 7.0 for data analysis. A p value < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

After the initial extraction procedure, from the aerial parts of *A. crucigerum* 36.8 g of methanolic extract were obtained (7.35% yield). For *A. paniculatum*, 49.9 g of extract were obtained (9.98% yield).

The methanolic extracts were submitted to an extraction procedure as described previously. Five fractions were obtained for each plant. The yields were as follows for *A. crucigerum*: hexanic 11.2%, chloroformic 20.3%, ethyl acetate 3.2%, butanolic 16.9%, and aqueous 22.1%. For *A. paniculatum*, the yields were: hexanic 1.11%, chloroformic 4.15%, ethyl acetate 44.7%, butanolic 12.51%, and aqueous 10.9%.

As result of the preliminary phytochemical tests, in *A. crucigerum* were detected alkaloids, tannins, saponins, and triterpenes/steroids. In *A. paniculatum* were detected flavonoids, saponins, and triterpenes/steroids.

The results of the evaluation of the anthelmintic activity of the crude extract and fractions of *A. crucigerum* are shown in Figure 1.

The evaluation of the anthelmintic activity of the crude extract and fractions of *A. paniculatum* gave the results shown in Figure 2.

The evaluation of the anthelmintic activity showed that the crude extracts were more active than albendazole regarding the times of paralysis and death. *Amphilophium crucigerum* methanolic extract showed an intense anthelmintic effect as it can be observed in Figure 1A. The Tukey *post-hoc* test showed that, regarding

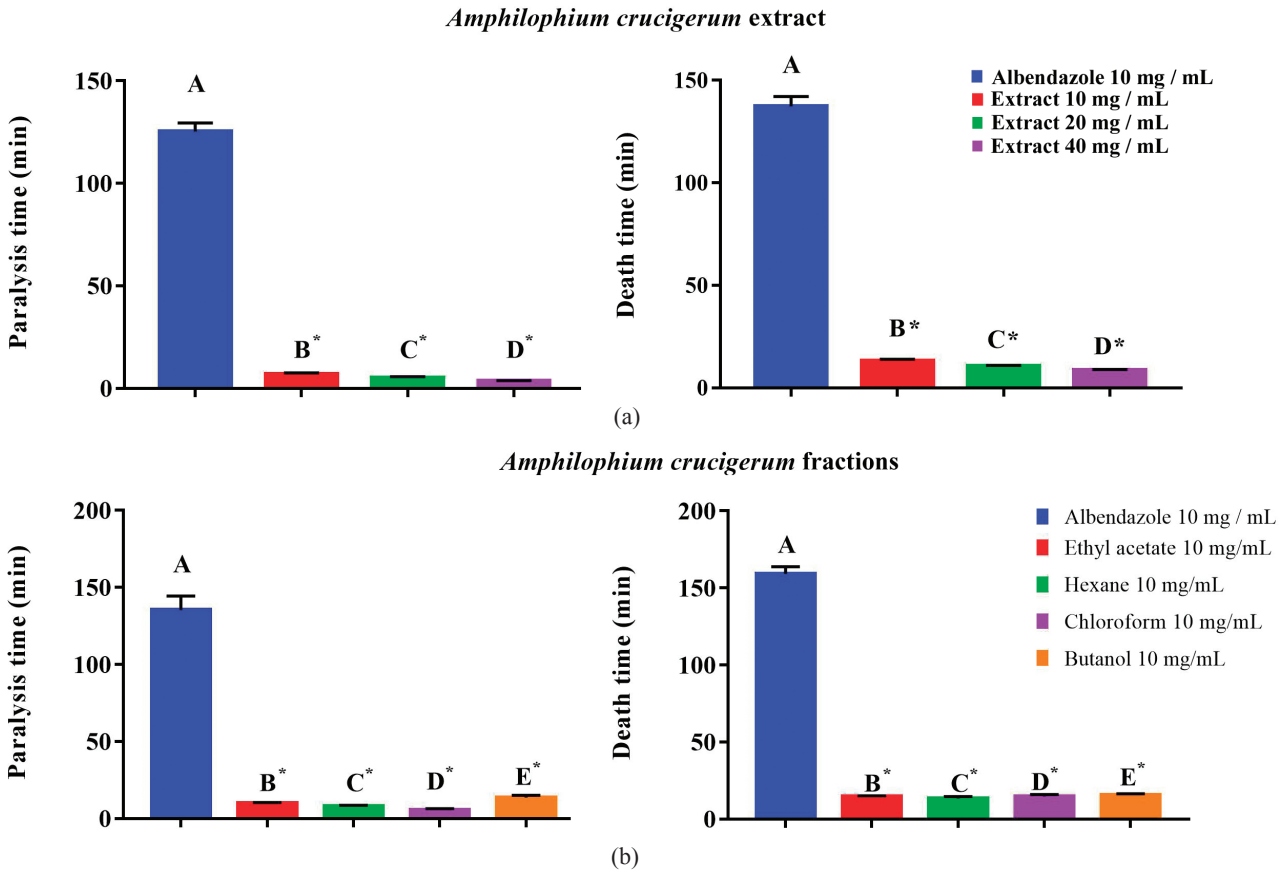

Figure 1: (A) Anthelmintic activity of the crude extract of *A. crucigerum* against *E. fetida*: *Y* axis is time for paralysis and time for death (in minutes). The values were expressed as mean ± standard deviation, and bars marked with different letters show statistically significant differences with respect to albendazole with $p < 0.05$ (ANOVA, Tukey). (B) Anthelmintic activity of the fractions of *A. crucigerum* against *E. fetida:* Y axis is time for paralysis and time for death (in minutes). The values are expressed as mean ± standard deviation, and bars marked with different letters show statistically significant differences with respect to albendazole with $p < 0.05$ (ANOVA, Tukey).

the time of paralysis, statistical significant differences only exist between the 10 and 40 mg/ml concentration, indicating that the 20 mg/ml concentration is as active as the two others. For the time of death, nevertheless, statistical significant differences exist between the three concentrations assayed, indicating an increase in lethality as concentration raises. The action starts quickly and takes approximately 7 minutes to paralyze the worms at the lowest concentration tested (10 mg/ml). This fact indicates that very active compounds are present in the extract. The time of death is also short (13 minutes for the lowest concentration).

In the case of the fractions of *A. crucigerum*, the aqueous showed to be inactive related to the control, but the other four were active compared to albendazole as shown in Figure 1B. In relation to the time of paralysis, the chloroformic and hexanic fractions were more active than the butanolic and the last one were as active as the ethyl acetate as no statistical significant differences were observed between the last two according to Tukey *post-hoc* test. The time of paralysis of the hexanic and chloroformic fractions was very similar to that of the crude extract. The aforementioned data could indicate that the more active substances are concentrated in those fractions, which should be analyzed separately to isolate the individual components. With respect to the time of death, all the fractions were more active than the positive control and there is no statistical difference among them. The death of the worms is quick, taking 13 to 15 minutes to kill all of them. The times are very similar to those obtained with the crude extract.

Amphilophium paniculatum crude extract showed also better anthelmintic activity than albendazole. The effect is concentration-dependent, as can be observed from Figure 2A. Although this is a crude extract, a concentration-dependent activity is desirable, because it would permit a better control of the effect produced than a concentration-independent one [16]. Nevertheless, the active compounds still has to be isolated and assayed in order to determine if the behavior of the crude extract is a reflection of those of their individual components. The same tendency is observed regarding the time of death, as can be seen in Figure 2A. There are statistically significant differences between all the concentrations tested for paralysis and death times, with each other and with respect to the positive control.

The aqueous fraction of this plant proved also to be inactive, similarly as observed for *A. crucigerum*. It appears that highly polar water soluble compounds present in both plants are devoid of anthelmintic activity. The more active fractions were the hexanic and butanolic and the chloroformic was the less active as shown

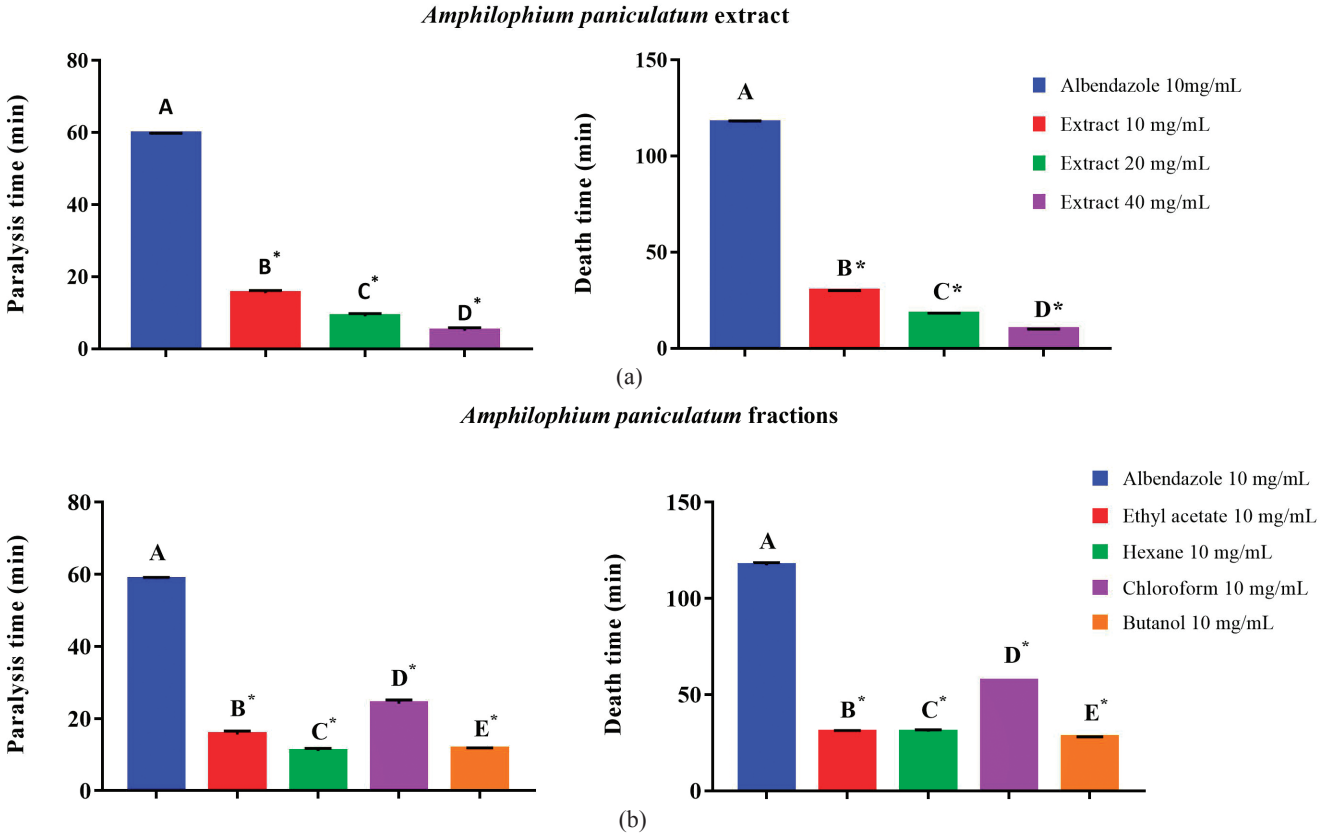

Figure 2: (A) Anthelmintic activity of the crude extract of *A. paniculatum* against *E. fetida*: Y axis is time for paralysis and time for death (in minutes). The values were expressed as mean ± standard deviation, and bars marked with different letters show statistically significant differences with respect to albendazole with $p < 0.05$ (ANOVA, Tukey). (B) Anthelmintic activity of the fractions of *A. paniculatum* against *E. fetida*: Y axis is time for paralysis and time for death (in minutes). The values are expressed as mean ± standard deviation, and bars marked with different letters show statistically significant differences with respect to albendazole with $p < 0.05$ (ANOVA, Tukey).

in Figure 2B. Significant differences were observed between all the fractions with respect to the positive control and to each other, except for the hexanic as compared with the butanolic. This results contrast with those obtained for *A. crucigerum* fractions. One possible explanation is that the chemical composition of both plants is not the same and this fact could influence the activity observed for the fractions. The behavior regarding the time of death for *A. paniculatum* fractions is similar as compared to the time of paralysis, except that the ethyl acetate fraction is as active as the hexanic as no statistical differences were observed between them.

4. CONCLUSION

The extracts and fractions of *A. crucigerum* and *A. paniculatum* possess compounds that have anthelmintic activity. The effect is more intense than that of the control drug, albendazole. The isolation and assay of the individual active substances will permit to obtain leading compounds for the development of new anthelmintic drugs.

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CONFLICT OF INTEREST

None.

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